CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY
RESPIRATORY AND CARDIOVASCULAR SYSTEM

Fourth Conference

ORGANIZED BY

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NOVEMBER 1-3, 2012

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Non-Traditional Biomarkers: Challenges & Applications of Biomarkers in Safety and Translational Pharmacology

STP I meeting, Nov 1-3, 2012

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Pfizer Internal Use

Overview

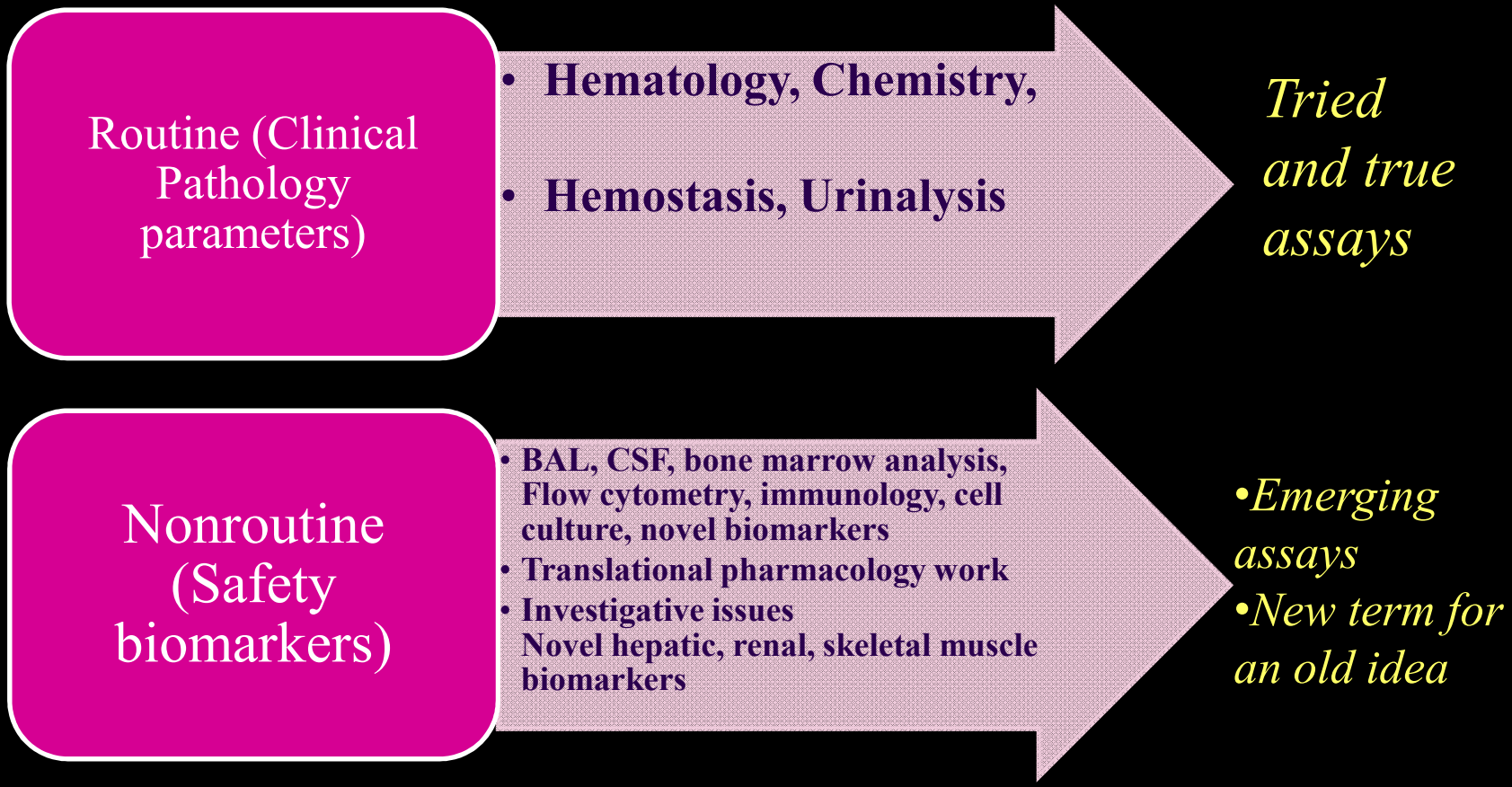
Part 1: Background

Clinical Pathology, Novel biomarker Challenges

- **Part 2: Cardiac Injury Biomarkers**
- **Part 3:** Biomarkers for both "safety" and "translational pharmacology" applications
  - Kidney BM
  - Cytokines & Acute phase proteins

Case examples

Clinical Pathology versus Biomarkers?



An old "routine" assay can be a "new" biomarker if used differently

Why do clinical pathology parameter testing in pre-clinical safety studies?

Key goals of toxicology studies to support clinical trials

- ***Identify an initial safe dose and the dose escalation regimen***
- ***Identify potential target organs and reversibility***
- ***Identify safety parameters for clinical monitoring***

Major components of a Tox-study

- *In-life*
- *Clinical pathology*
- *Anatomic Pathology*
- *TK*

In-life method to evaluate for toxicity (exaggerated pharmacology) in the following organ systems

- Hematopoietic (blood and bone marrow)
- Hepatic
- Renal
- Musculoskeletal
- Cardiovascular
- Endocrine
- Immune
- Mineral/Electrolyte metabolism

Contextual linkage to other study data

Translation of Safety Biomarkers in Drug Development; Difficult to monitor areas

Testicular biomarkers

Glomerular Injury biomarkers

Vascular injury biomarkers

Drug Induced Liver Injury (Idiosyncratic responses)

Testicular Toxicity Biomarker Gap

Preclinically, most sensitive method for detection of testicular toxicity is histopathologic assessment of the testis and epididymis.

Other quantitative methods include epididymal sperm counts and motility, testicular spermatid counts, and measuring sex and/or gonadotropin hormones level in blood.

Inhibin B has emerged as a biomarker of testicular toxicity for possible use in preclinical and clinical studies although there are no extensive studies for its validity.

New translatable biomarkers of testicular damage is a significant need.

Lack of Glomerular injury Biomarkers

- Glomerular injury associated with large molecules
  - (Lack of translatable, monitorable glomerular injury biomarkers that can be employed in clinic once preclinical signal is identified)
- Glomerular injury with small molecules may be rare compared to tubular injury
- Novel set of biomarkers with better sensitivity than albuminuria is needed that will track glomerular injury caused by drugs
  - Enable increased TI, monitorability and reversibility

Hepatotoxicity Biomarker Issues

	Biomarkers that identify hepatic function	*Biomarkers that can test mechanisms*	*Biomarkers that can predict idiosyncratic DILI*	*Biomarkers that address nonspecific ALT elevations*
Significant benefits	▪ Better hepatic functional biomarker will test if the signal is related to impending hepatic failure	▪ Information on the mechanistic basis of injury will result in potential risk management	▪ Will help address patient monitoring and can be used to mitigate DILI development	▪ Improve specificity and provide value for continued therapy
Current state	▪ Bilirubin pre-clinically of less value ▪ PON1 shows some promise but not yet qualified	▪ miRNA may be of potential utility, but still in early stages	▪ No promise yet with serum markers ▪ Recent studies have shown promise with genetic HLA screening	▪ PPMG biomarkers show some promise but not prospectively validated ▪ New biomarkers still in exploratory stages

Hepatotoxicity is costly safety issue for pharmaceutical companies either in terms of withdrawal from the market (Rezulin®) or severe labeling restrictions (Trovan®) or attrition in nonclinical or clinical development.

Biomarker translation; Some are easy and some complex!

EASY

Glucose

LESS OBVIOUS BUT ACHIEVABLE……

Coagulation markers

UPHILL, ROCKY AND CHALLENGING…..

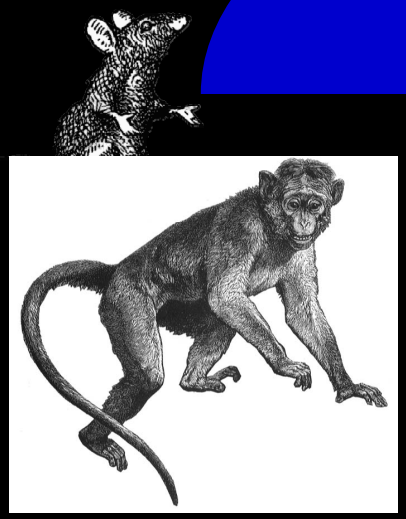
Idiosyncratic liver injury

Glomerular injury

Hypersensitivity

Translation of mechanisms, assays and timing critical

Hurdles for Translation: Major Buckets



Models

Biomarker performance

Patho-Mechanisms

Assays

Practicality

Systematic approach and multidisciplinary collaboration critical for translating safety biomarkers into clinics and as diagnostic tests

Animal model as an hurdle for translation?

- Human studies mirroring the preclinical toxicity studies generally cannot be conducted
  - Treatments with a wide variety of known toxicants is not possible
  - Regular histopathology (i.e. biopsy) of target organs would not be practical
  - Pathologic changes are usually not similar across studies
  - Preclinical and clinical scientists need to align on preclinical studies designs
  - Difficulty to generate relevant animal models
  - Are we using rats or non rodents (fewer NHP's and dogs compared to rats)?

Predictability of Animal Testing

- Why are animals studies poorly predictive for idiosyncratic reactions?
  - Relatively few animals tested compared to humans exposed in clinical trials
  - Large clinical trials needed for DILI compared to relatively few animals preclinically
  - May be idiosyncratic only in one species
  - May be idiosyncratic in all species

Olson H et al. Regul Toxicol Pharmacol. (2000) 32: 56-67. Concordance of the toxicity of pharmaceuticals in humans and in animals.

Biomarker performance as hurdles for translation

- Complications with assessing biomarker performance in human studies
  - Benchmarking against histopathology is generally impractical
  - Benchmarking against current but flawed biomarkers (e.g. urine microalbumin, ALT, BUN) is complicated
  - Benchmarking against an infrequent clinical outcome may indicate many “false positives” for a sensitive biomarker of injury
  - Monitoring biomarker performance in human disease that approximates drug-induced injury
  - Monitoring biomarker performance in standard treatments that are known to carry a risk of injury:
    - Halothane hepatotoxicity
    - Acetaminophen hepatotoxicity
    - Cisplatin testicular toxicity
    - Tacrolimus or cyclosporine A treated patients

Patho-mechanism as hurdle for translation

- Similarities and differences may exist in mechanisms across species
  - Physical, metabolic and pathologic changes; eg; is hematopoiesis different across species? T1/2 of RBC's, neutrophils, platelets??
  -
  - Fairy conserved mechanisms and processes may translate better (eg; hematotoxicity)
  - Ability of the liver to regenerate following hepatic insult? Is there difference between rat, mice and human?
    - Hy's law is difficult to reproduce preclinically?
  -
  - Poor prediction for idiosyncratic responses

Logistical Translation Challenges?

- •Will need same tissue or fluid in clinical situations
- •Research labs are not equal to clinical space, may include multiple clinical sites
- •Limited or no technical support, protocol changes difficult
- •Impact on enrollment
- •Assay robustness and cost
- •IP/FTO
- •Reference ranges (challenging in clinical space)
- •Magnitude of change for clinical relevance
- •Impact on enrollment

Additional challenges for clinical Translation of New Safety Biomarkers

- Clinical themes
  - Determination of baseline biomarker values
  - Determination of prognostic / diagnostic threshold values
  - Testing of threshold values
  - Testing clinical outcomes based on biomarker-driven interventions

Part 2: Safety Assessment of Cardiac Toxicity

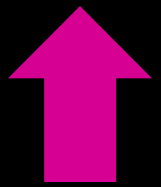
Traditional Approach

Δ Function

Hemodynamics
Electrophysiology

Δ Structure

Heart

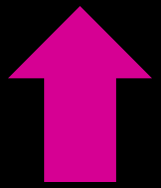


Safety Pharmacology

In vitro K^+, Na^+, Ca^{2+}
In vivo telemetered animal studies

Pathology

Gross and microscopic evaluation in repeat dose studies

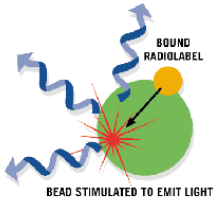


Biomarkers

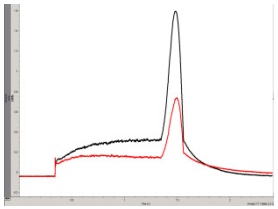
In vitro QT assessment (dofetilide and hERG)

Dofetilide binding assay is designed for higher throughput screening of compounds for SAR.

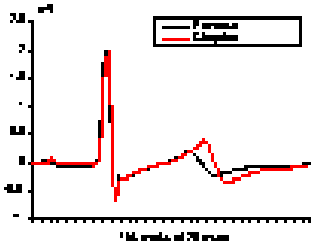
Study Purpose:
To define the potential for a compound to interact with the hERG potassium channel and have potential to delay cardiac repolarization (QT prolongation).



Dofetilide

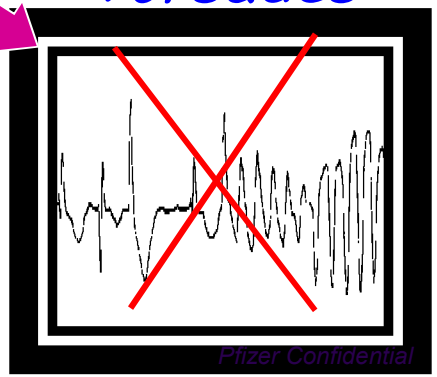


hERG



In vivo

Torsades



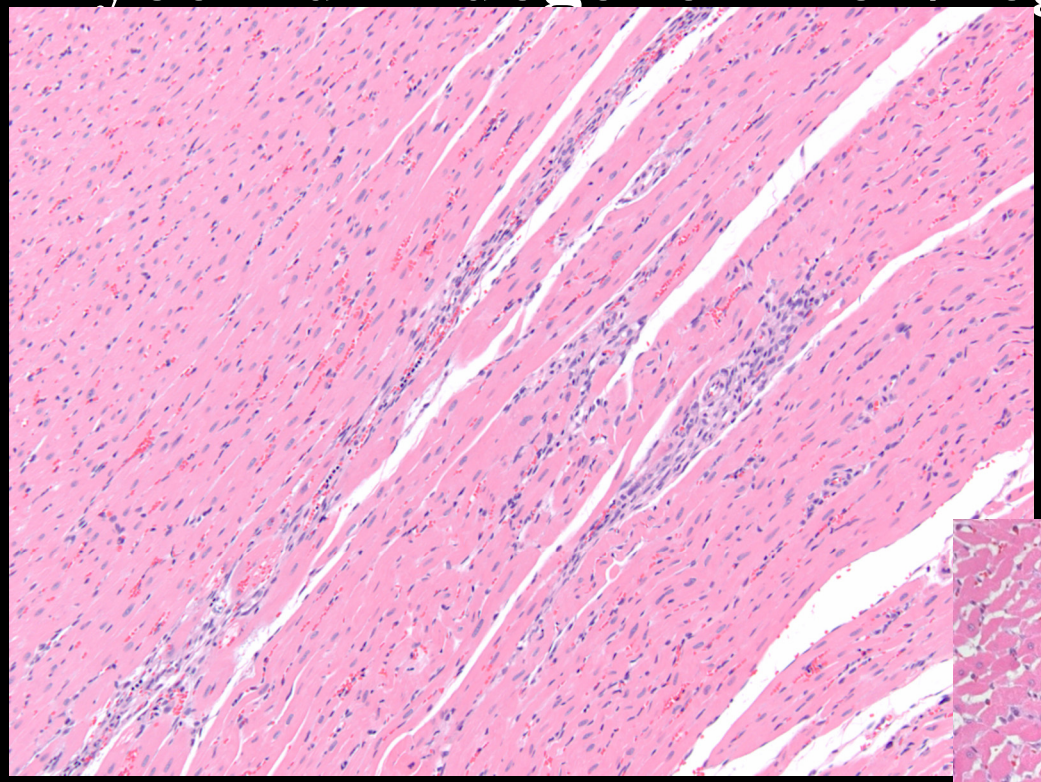
- **hERG is used to generate a safety margin (~300x between hERG IC50 and project Ceff in humans), usually around lead selection, based on the estimated efficacious free plasma concentration in humans**
- **Derisking hERG is high priority due to potential QT prolongation. Significant concern for clinicians and regulators with this liability**
- **Anesthetized Guinea pig infusion studies at ~30X margin**

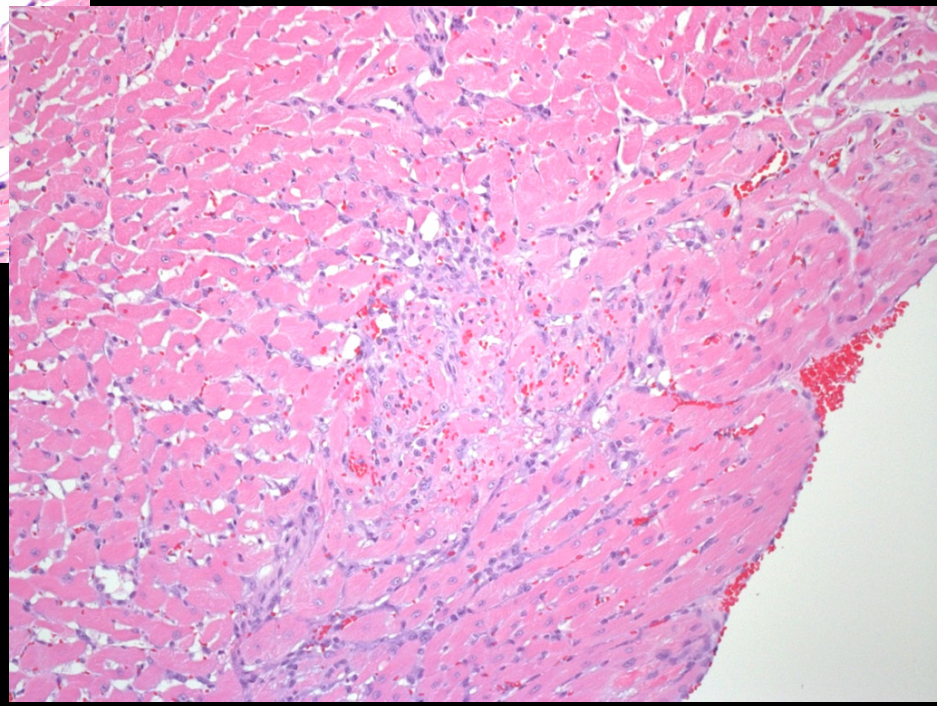
Pfizer Confidential

Current state of Biomarkers and gaps

- **Current CV biomarkers**
  - HR and BP - well established functional markers
  - LVP dP\dT – measure of cardiac contractility (echocardiography)
  - Troponins (TnI isoform specific for cardiac muscle)
    - used clinically – well accepted
    - Works across species; Ultra sensitive Singulex-assays available – concerns with over sensitivity
  - BNP - used clinically as a marker of hemodynamic stress, dog assay exists
  - NT-proANP – becoming accepted as marker of congestive heart failure
- **Perceived Gaps**
  - Leading biomarker s of cardiomyocyte damage to detect injury while damage is reversible
  - Current markers generally "report" lethal injury rather than "predict" it
- **Specific toxicities we want to be better able to monitor/predict**
  - Direct cardiac muscle toxicity – **more predictive than C-TnI**
  - Cardiac hypertrophy: BNP, NTproANP
  - Valvulopathy

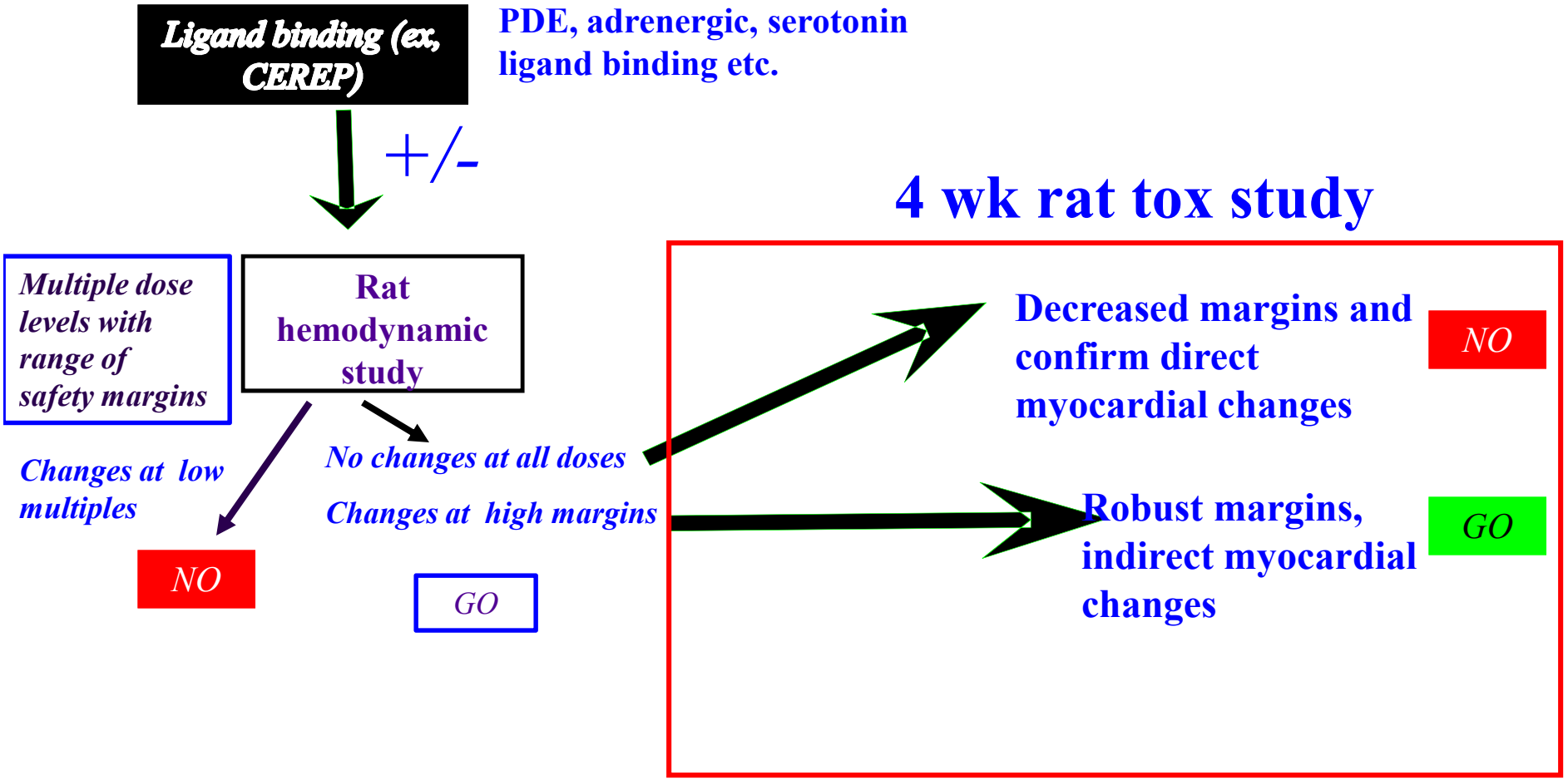
Myocardial degeneration/regeneration





HEART	:	5	5	5
- Degen/regen: myofib.	:	-	3	4
- Inflam myocardium	:	-	1	3

Derisking myocardial toxicity: Characterization example



- **Hemodynamic changes at higher doses may suggest myocardial damage due to hemodynamic changes (<u>indirect effect</u>)**
- **No hemodynamic changes suggest <u>direct</u> myocardial necrosis**

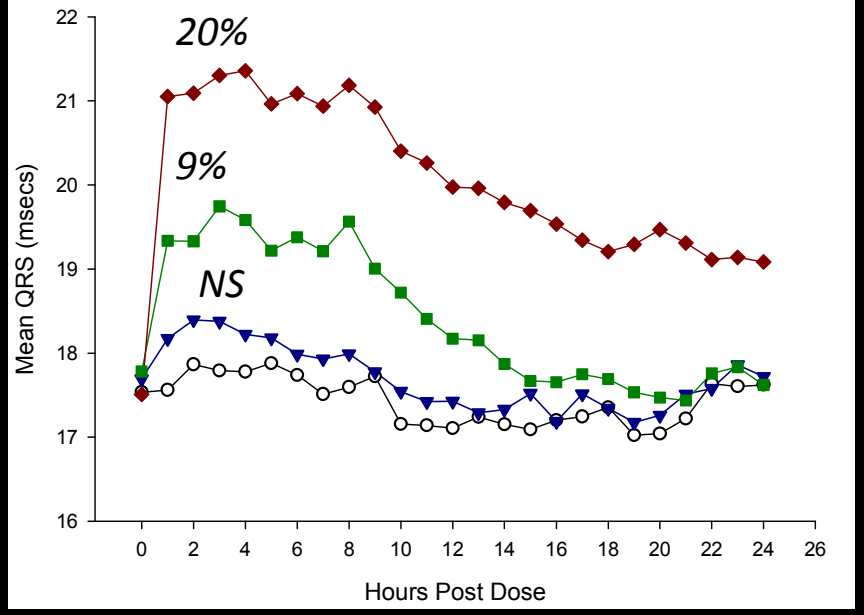
Case example

- **In vitro CV assessment**
- *hERG IC_{50}: 1.7 μM*
- *Therapeutic margin~200x*
- *Therapeutic Margin= hERG IC_{50}/Target IC_{50}*
- ***Further investigations into cardiovascular-profile***

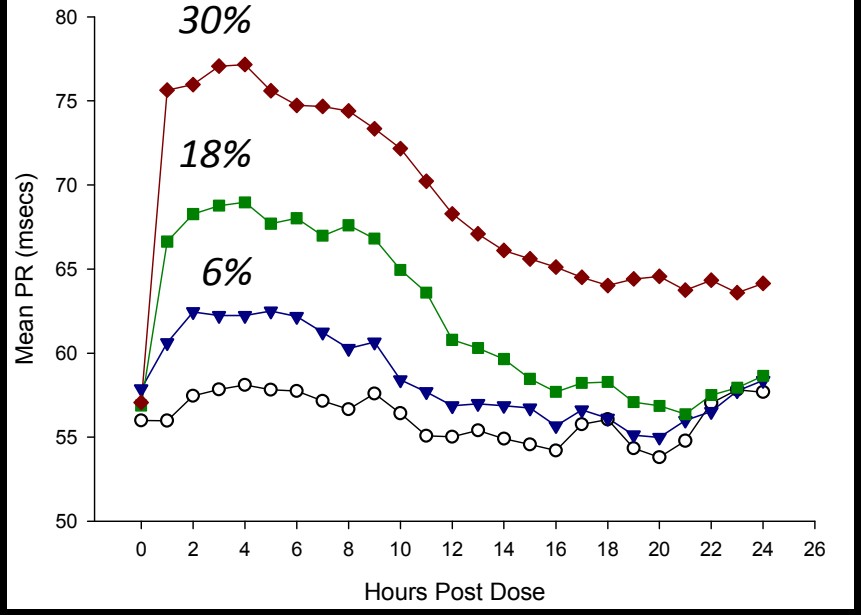
Langendorff and Rat in vivo CV Profile

- Lagendorff assay demonstrates statistically significant change in QT interval at 0.3 µM.
- Also reveals changes in PR and QRS intervals at similar concentrations.

QRS



PR



- *Percent changes at C_{max} following SD exposure rat.*

In vivo CV Profile: NHP

NHP CV GLP study results

Dose (mpk)	Multiple	CV Findings
Low dose	2	NOEL
Mid dose	6	↑QRS (6%)
High dose	19	↑PR(11%) & ↑QRS (12%)

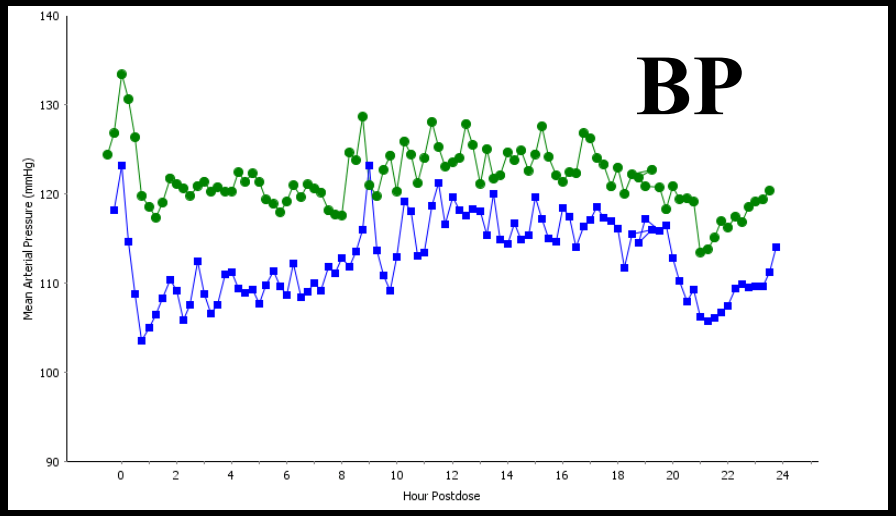
- Further evaluation revealed a PR and QRS interval changes in a NHP study at low multiples.

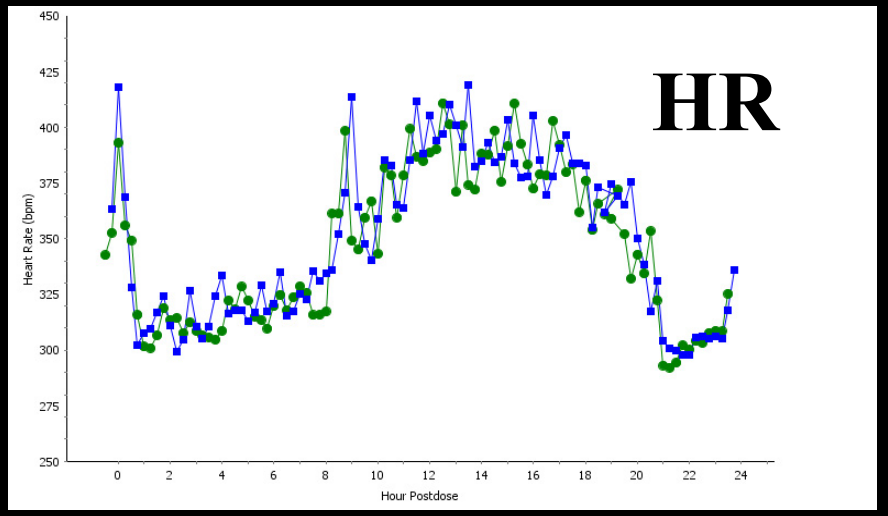
- Development limiting and compound termination

- QRS prolongation due to inhibition of sodium channel

- Langendorff model to screen new compounds to test sodium channel inhibition

Another example; Day 4 Results – Compound A: Rat telemetry data





- Compound A produced a rise in blood pressure up to 8 mmHg after a single dose which was biologically significant
  - Heart rate showed a trend towards lowering but did not reach the 25 bpm threshold generally considered as significant
- Repeat dosing produced a sustained elevation in blood pressure (6-13 mmHg) which lasted for the full 24h of postdose monitoring
  - HR was no different from control at day 3 and 4

- **Jacketed telemetry combined with tox study in the dog: No finding**

Part 3: Biomarkers for both "safety" and "translational pharmacology" applications

Reverse translation example?

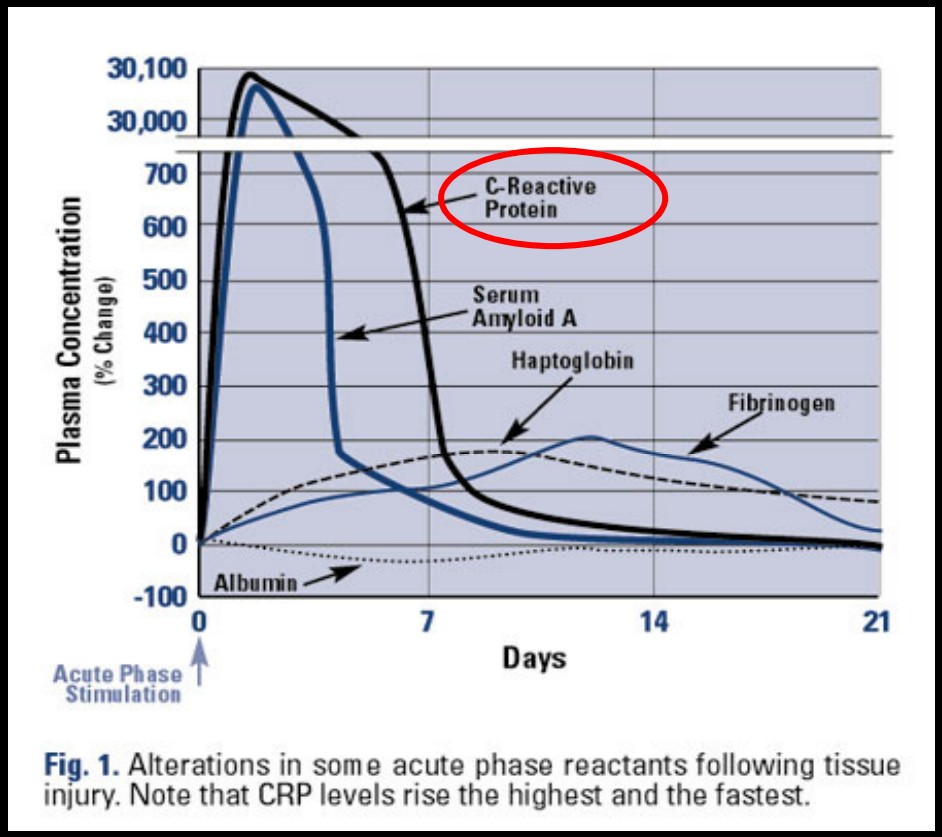


Fig. 1. Alterations in some acute phase reactants following tissue injury. Note that CRP levels rise the highest and the fastest.

Gabay, C., and I. Kushner. 1999. Acute-phase proteins and other systemic responses to inflammation. N. Engl. J. Med. 340:448-454.

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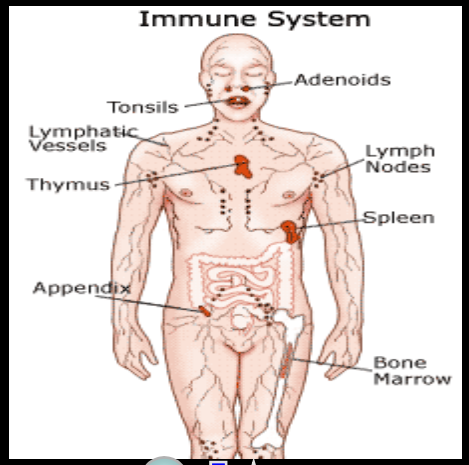
Current Gap

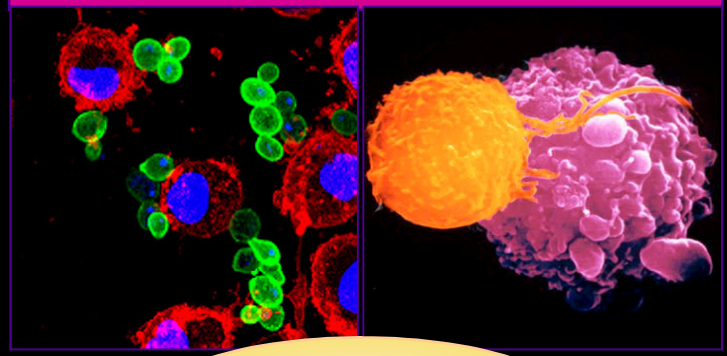
Disease (RA) and Inflammatory mechanisms



Innate Immunity

T and B cell activation

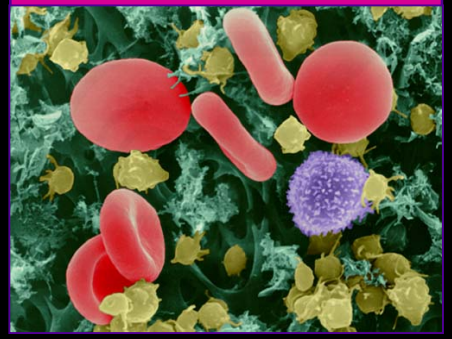
Normal Tissue Surveillance



Inflammatory cell trafficking

Immune cell effects on inflamed tissues

Circulation Blood/Lymphatic



Cytokines and mediators

Inflamed tissues

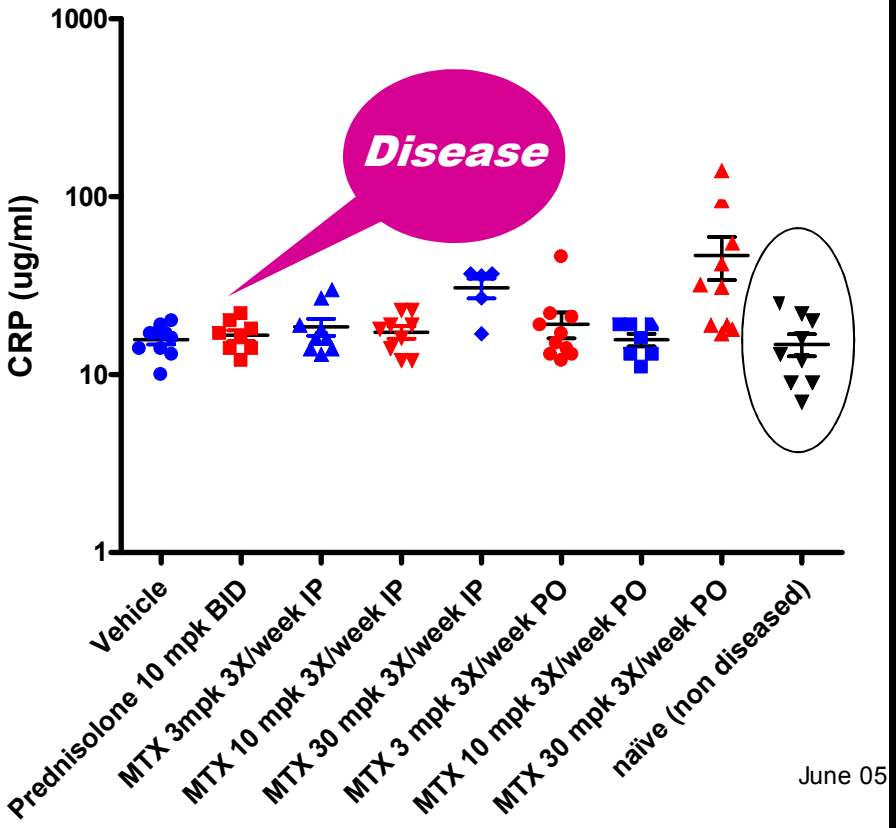
Species differences in utility of classic APRs as circulating markers:

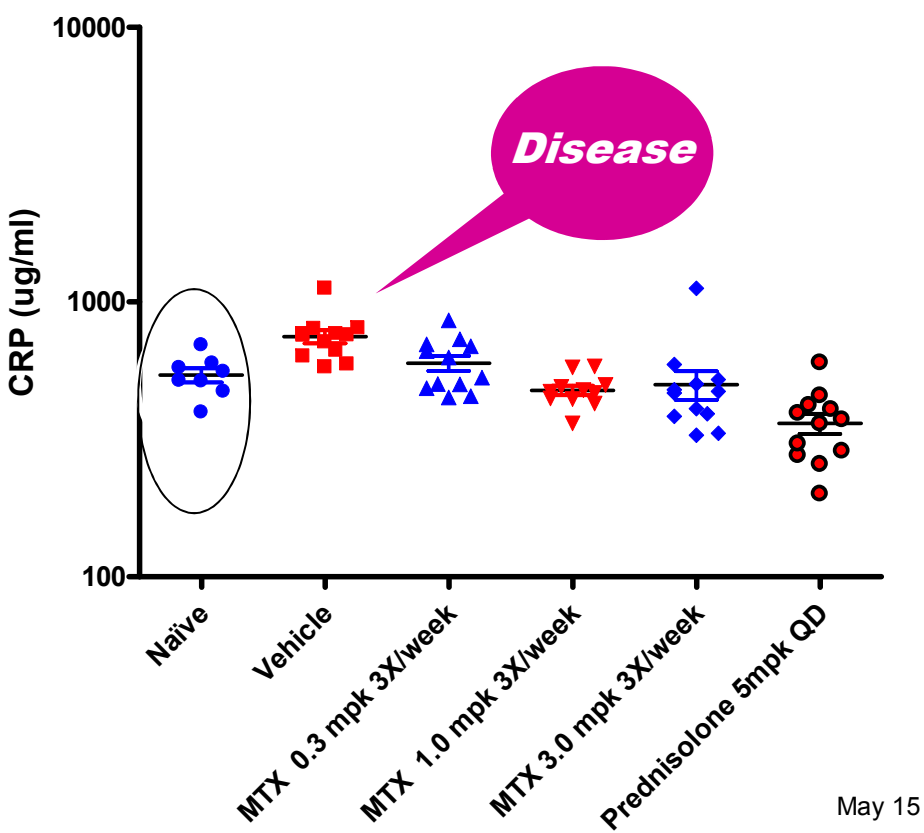
Species differences in APRs detectable in blood, and their responses

APR:	human	dog	rat	mouse	rabbit	Monkey
CRP	++++	++++	0	+	++++	++++
SAA	++++	++	0	++++*	++++	++++
SAP	0	0	0	+++	?	0
α2MG	0	+/-	++++	+/-	++	0
A1acid GP	++	++	+++	0	?	++
Ceruloplasmin	+	+	+	?	++	+
Haptoglobin	+	++	+++	++	+++	+
Fibrinogen	++	++	++	++	+++	++
Transferrin	↓	±↓	±↓	↓	↑	↓
↓alb	√	√	√	√	√	√
ESR	√	0	0	0	0	√

*+/- variable results; * strain-dependent + <1x, ++ 1-4x, +++ 5-10x, +++ >10x*

The Challenge: CRP doesn't translate to Rodent Models of Inflammation





Considerable Strain to Strain variation in the acute Phase Response

Mouse Strain	Acute Phase Response for SAP
C57BL	Lowest baseline SAP value with largest incremental response to Inflammation
DBA/2	Highest baseline SAP and least responsive to inflammation
BALB/c	Intermediate responder
C3H/He	Intermediate responder (endotoxin unresponsive strain)

Species	Strain
Mouse CIA	DBA
Rat AIA	Lewis
Rat CIA	Dark Agouti

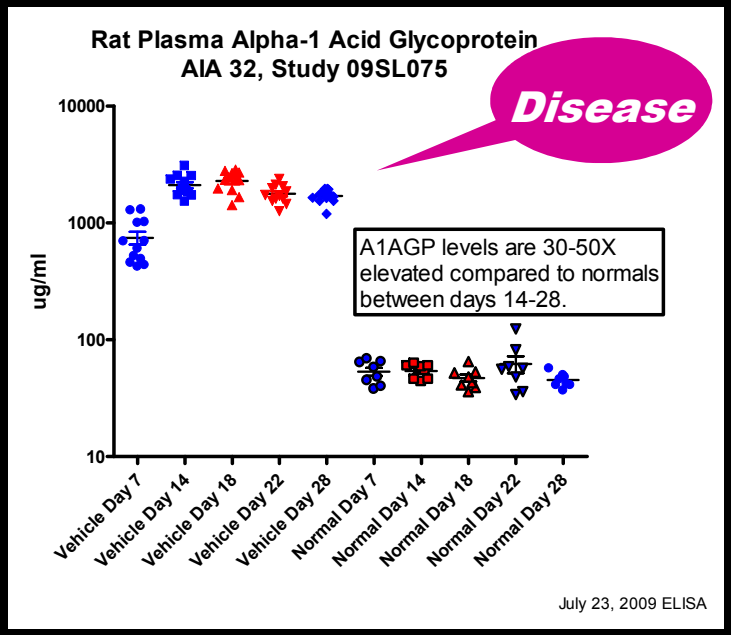
Data compiled in Biochemistry of Inflammation. J. T. Whicher, S. W. Evans. 1992.

Inflammation Models Utilize Multiple different Strains of Mice and Rats

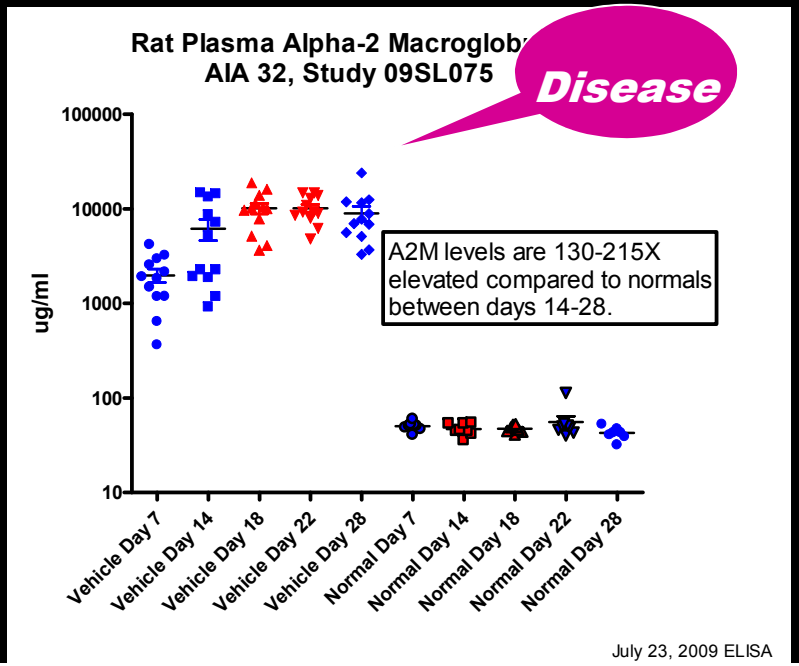
Species	Strain	Studies Examined	Relative Order of Response
Mouse CIA	DBA	5	SAA>SAP>CRP ++++ +++ +
Mouse TNBS	**BALBc**	**1**	**SAA>SAP** **++++ +++**
Rat AIA	Lewis	4	A2M>A1AGP>HA>CRP ++++ ++++ ++++ +
Rat CIA	**Dark Agouti**	**2**	**A2M>HA>A1AGP=CRP** **+++ ++ + +**
Rat TNBS 30mg/acute	**Sprague Dawley**	**4**	**A2M>HA>A1AGP>CRP** **++++ ++ ++ +**
Rat STZ	**Wistar**	**1**	**A2M, A1AGP barely elevated 11wks** **+ +**
Rat 5-Day IVT	**Wistar**	**1**	**A1AGP>A2M=HA** **++++ ++ ++**

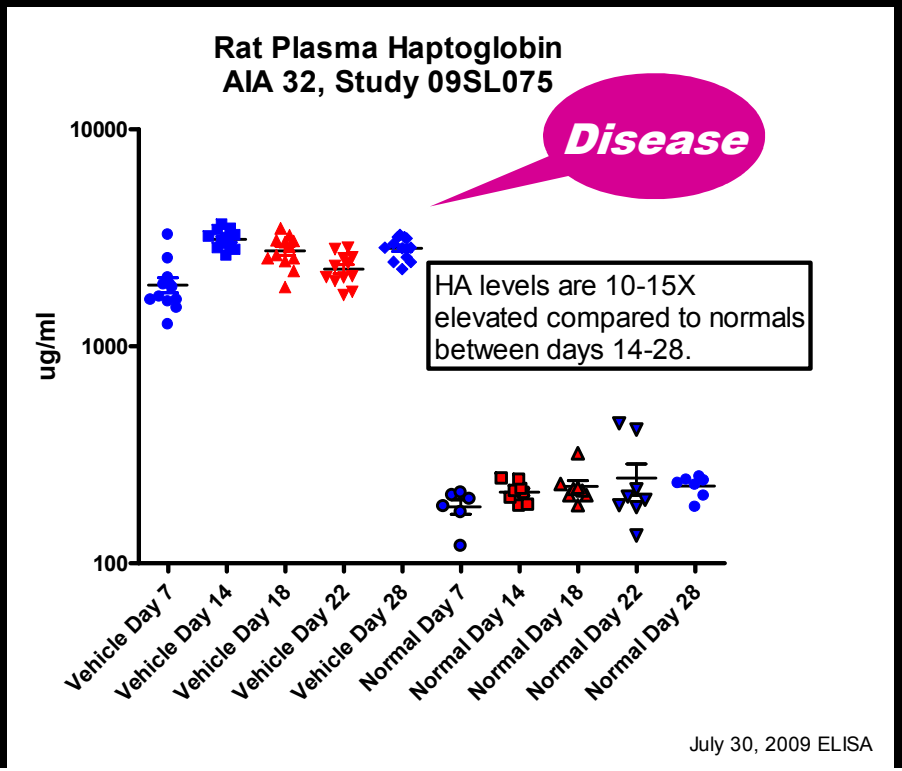
+ <2X, ++ 2-5X, +++ 5-10X, ++++>10X elevation

A2M, A1AGP, & HA are all significantly elevated in the Rat AIA model of inflammation

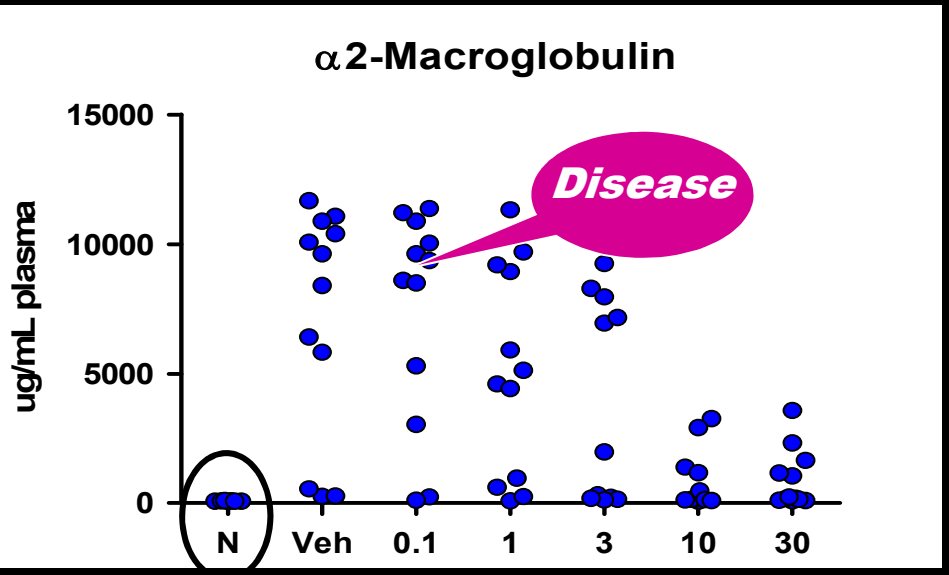


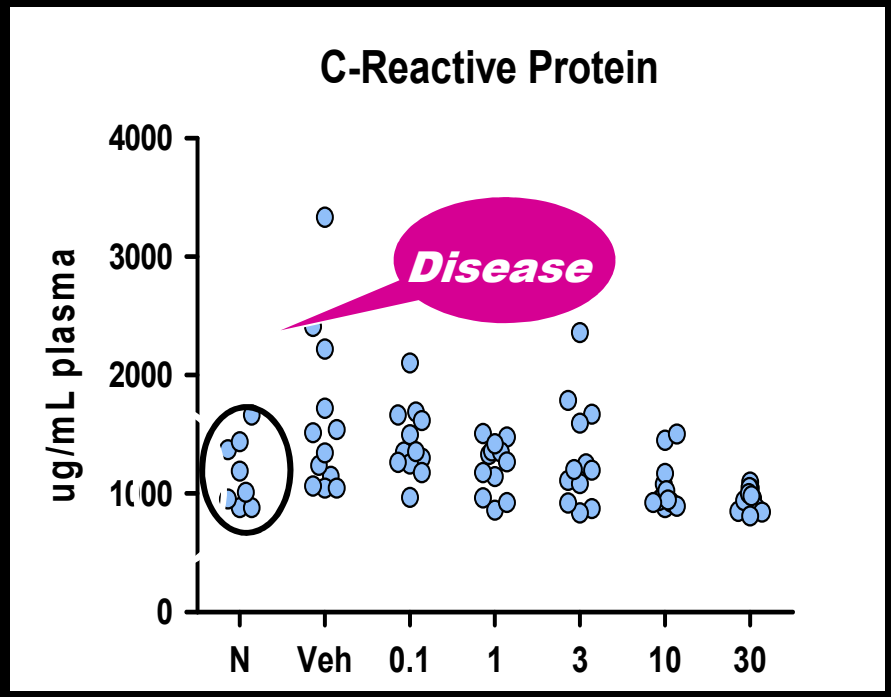
APP levels are still rising at day 7 in Rat AIA model, but level off between days 14-28 with magnitude increase A2M>A1AGP>HA. These windows should allow for a therapeutic dose response.

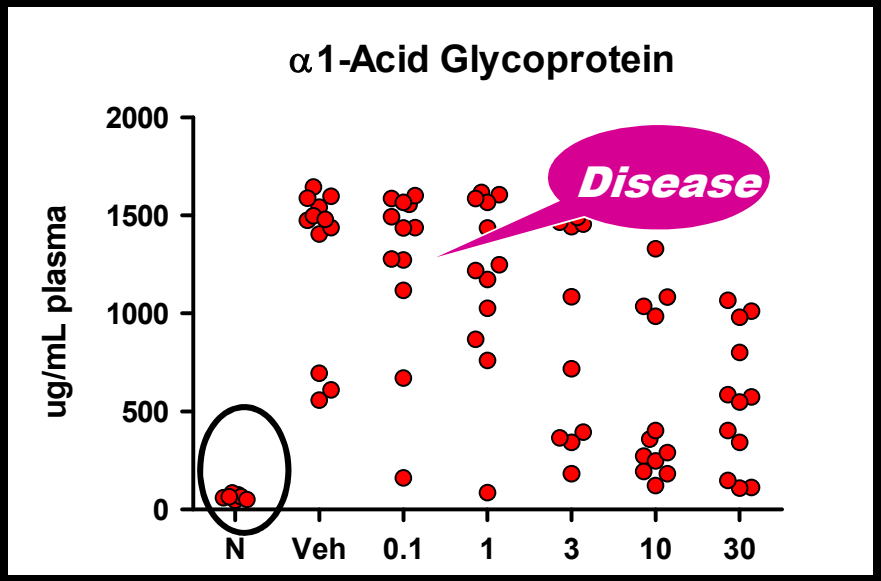


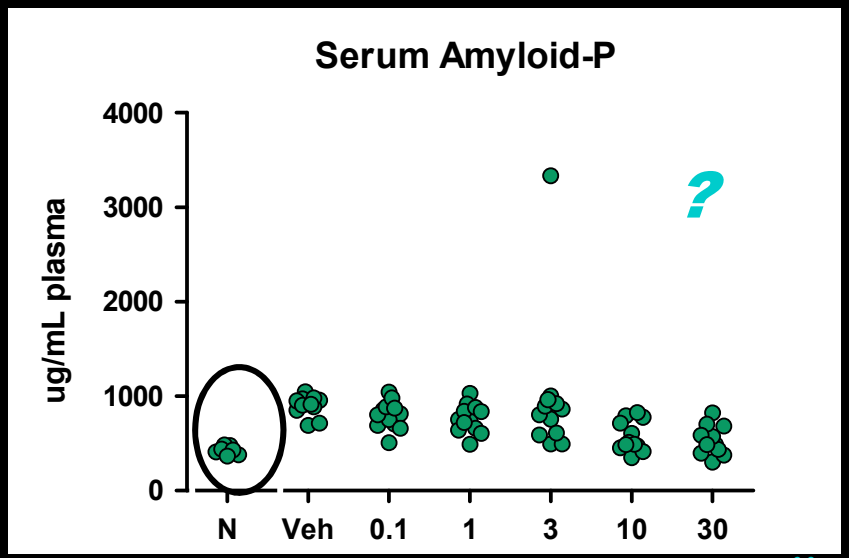


Rat AIA: Day 18 (w/dosing of Compound A)



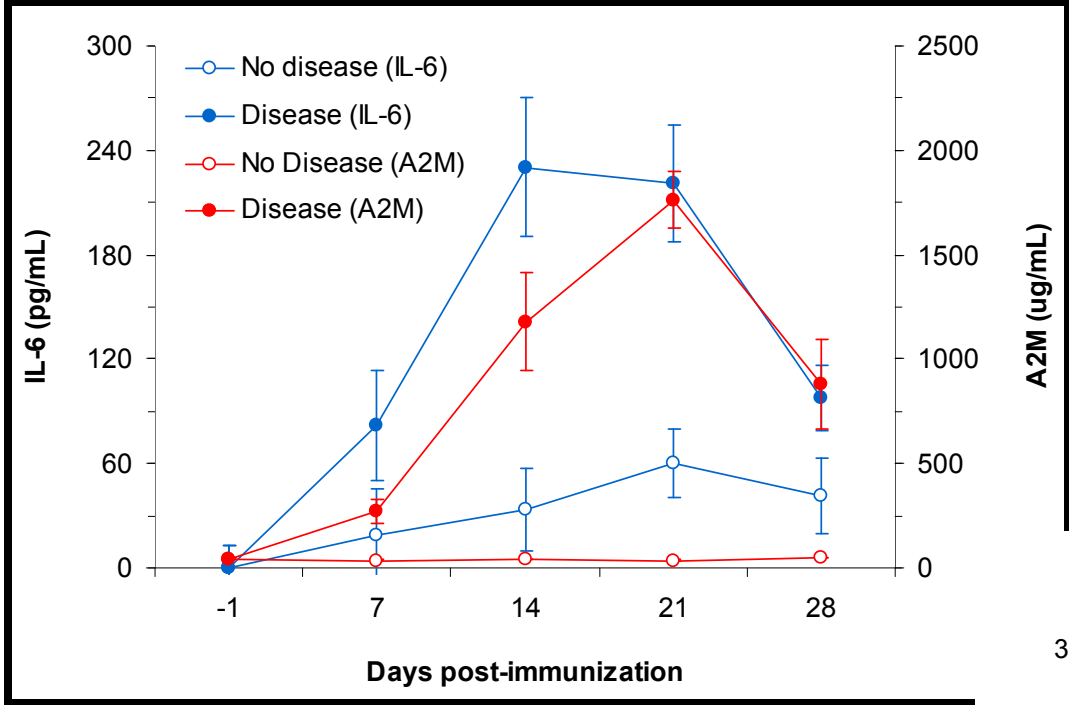


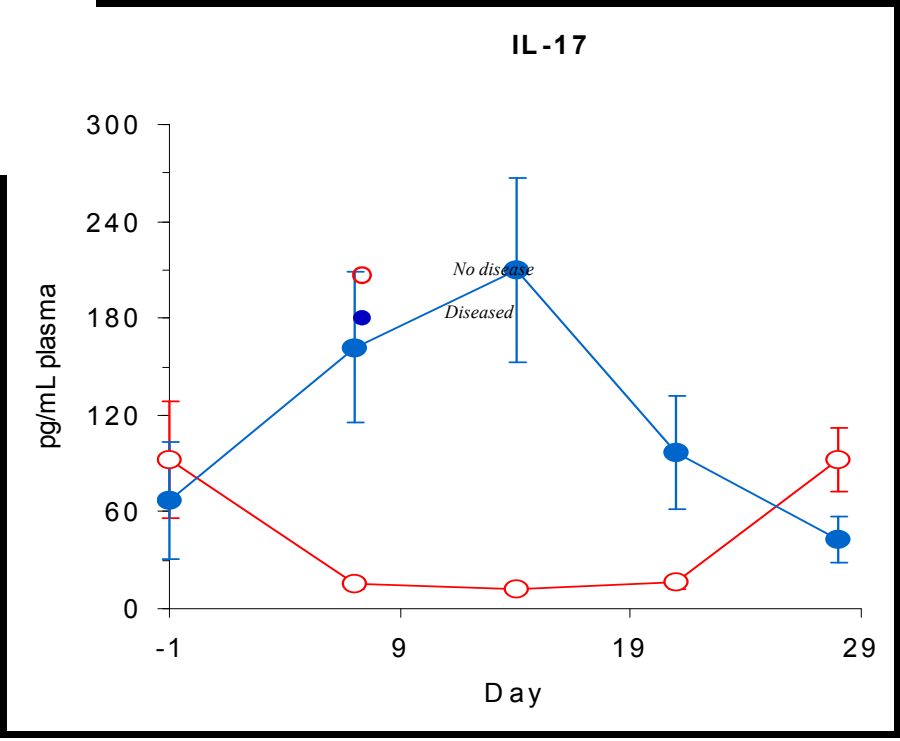




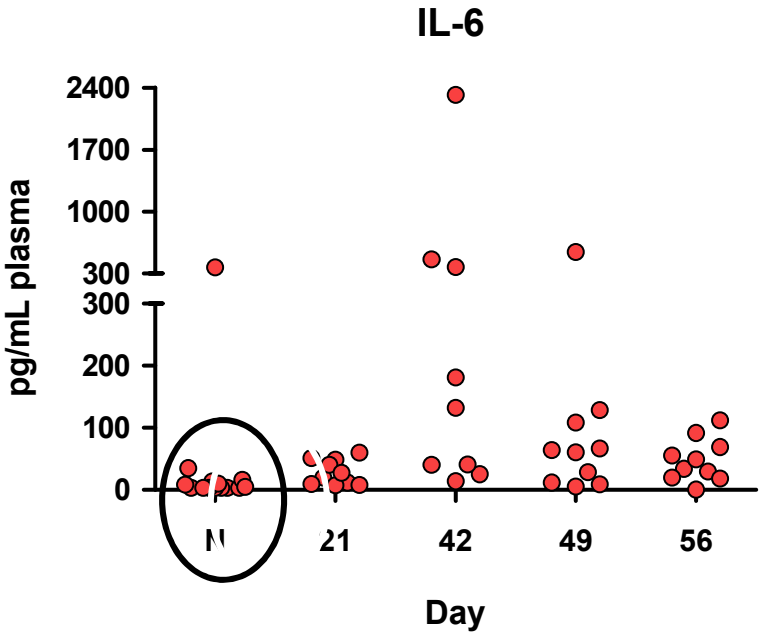
Note on cytokines

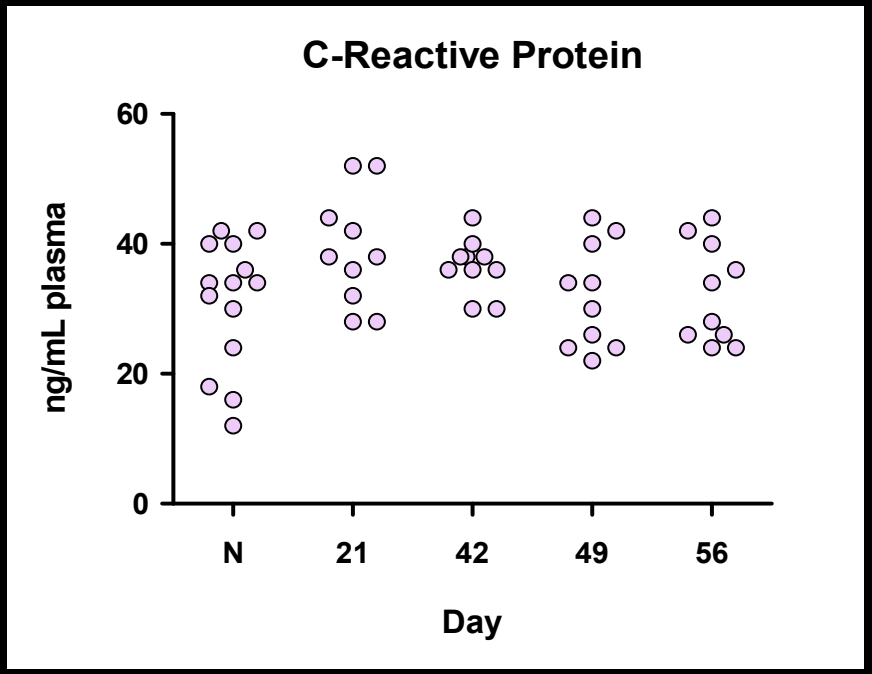
α-2-Macroglobulin, IL6, IL17 in Rat AIA Time-Course Study

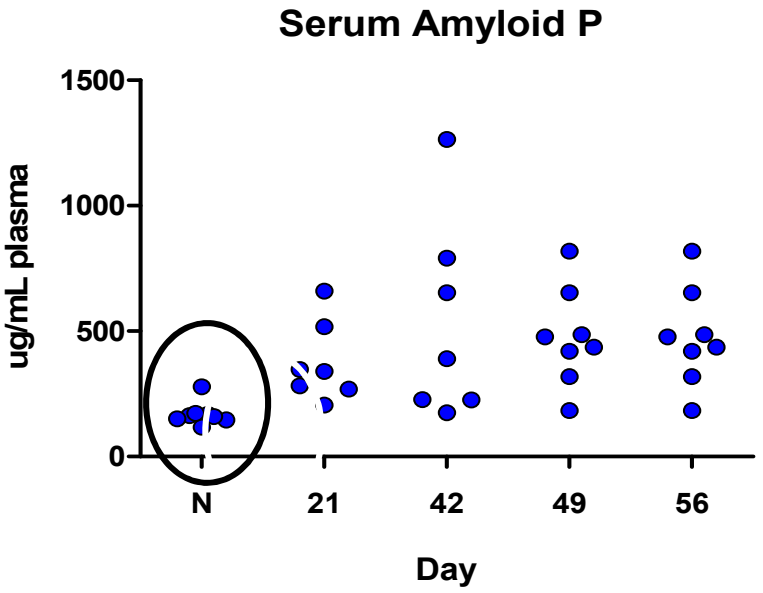


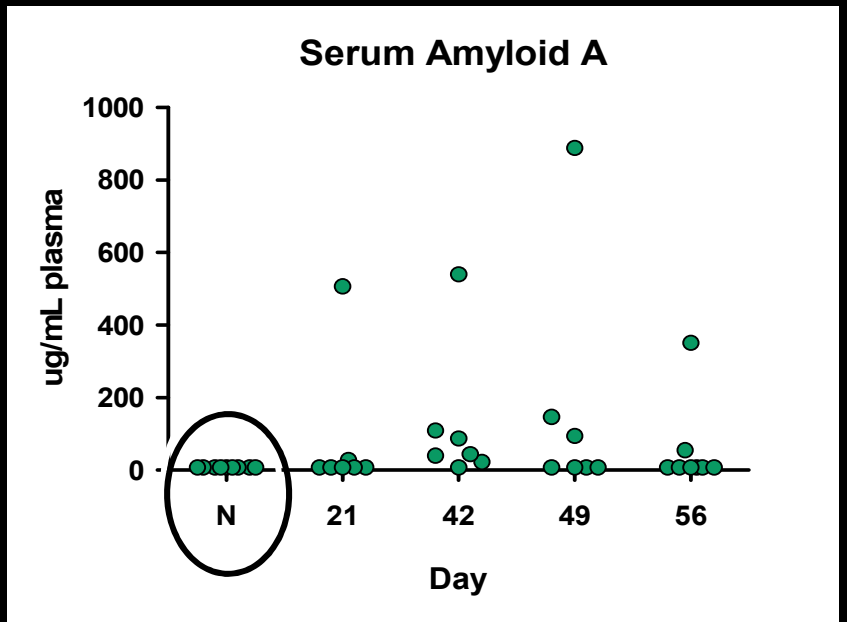


Traditional Mouse CIA

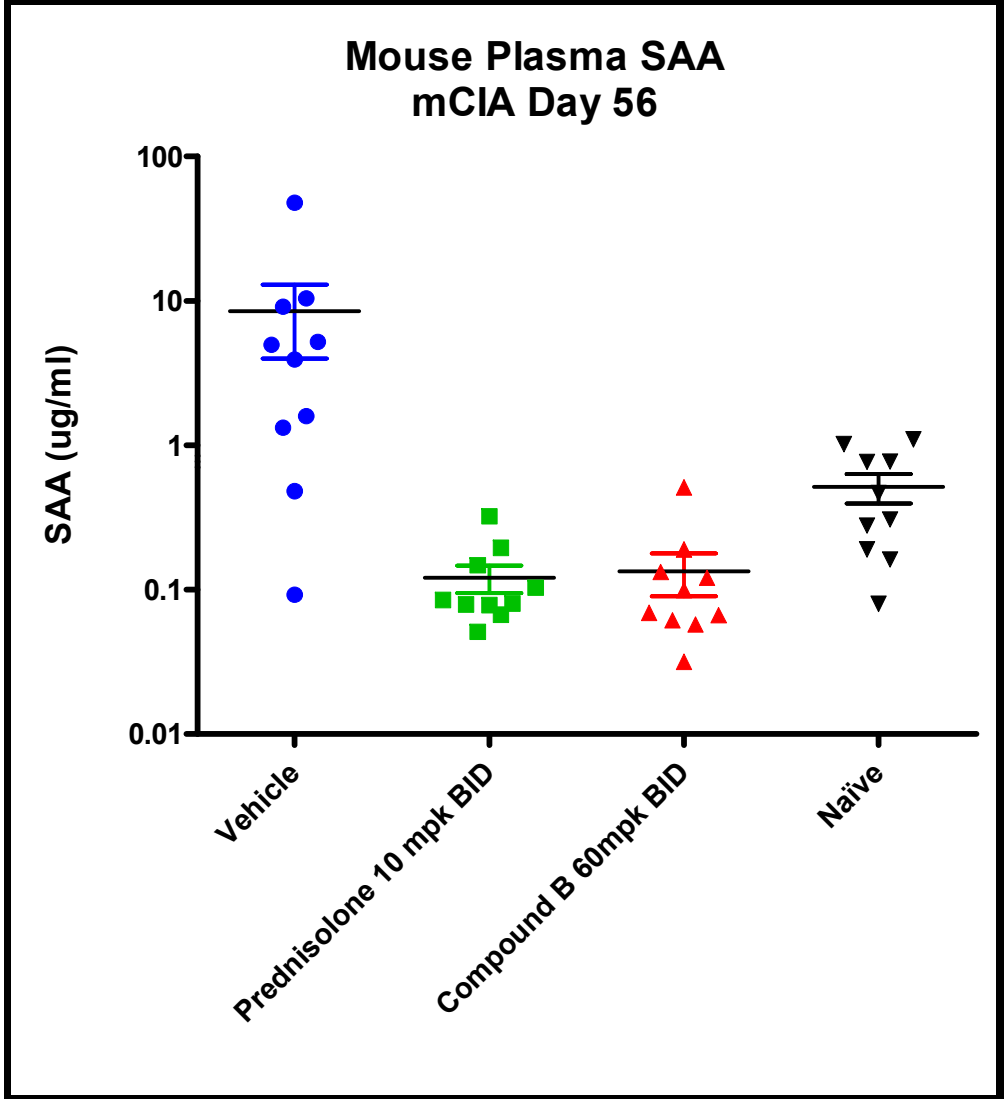


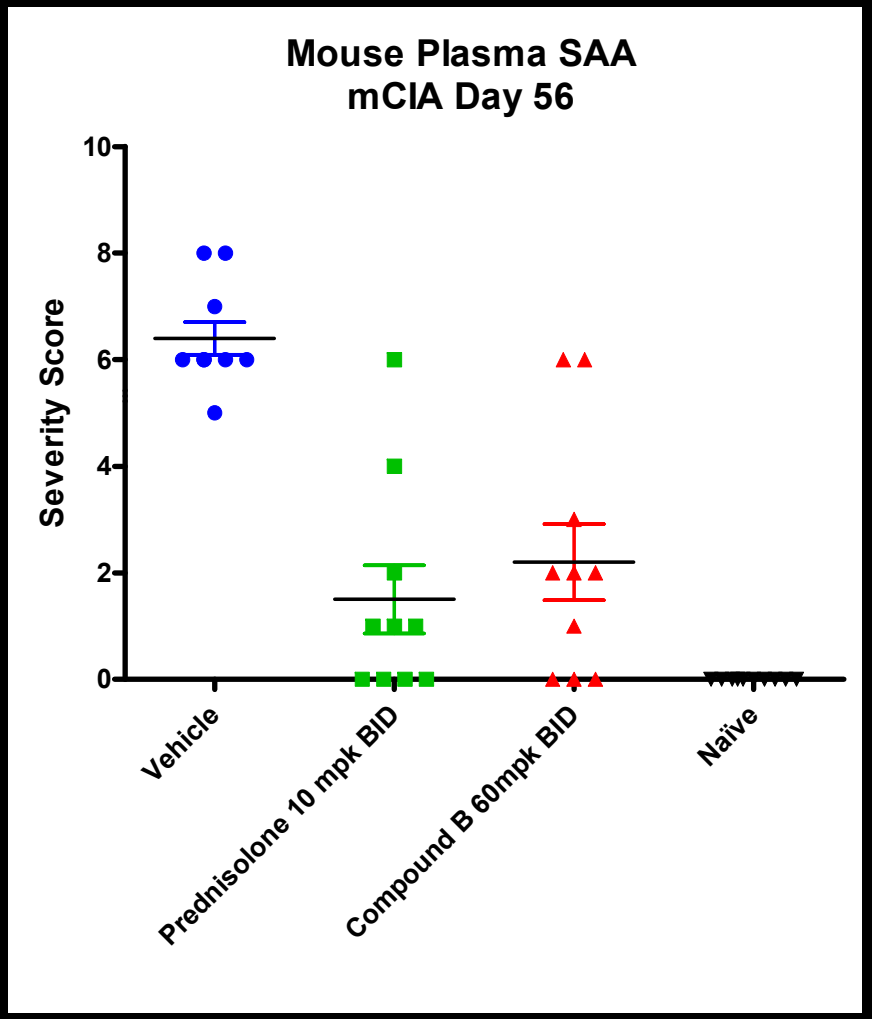






In the Mouse CIA Model, SAA decreases in Response to Therapeutic Dosing (D40) with either Prednisolone or Compound B Correlating with Efficacy





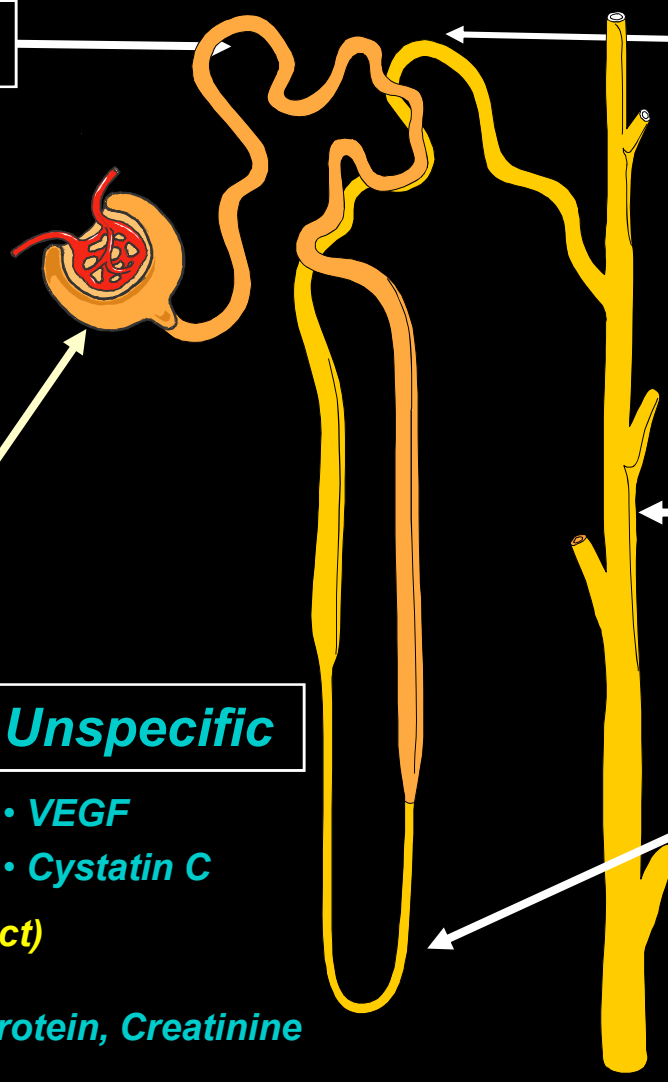
Acute Phase Proteins: Summary

- SAA and SAP for Mice and A2M, HA, and A1AGP for Rats
- Confirmed that CRP is not responsive to Inflammation in Mouse and Rat models.
- Initial data suggests that the APP response can correlate to disease, efficacy, and potentially toxicities or secondary pharmacology, but defining the appropriate APPs for some models is a work in progress, and additional data in models where efficacy is seen are necessary
- In Rat AIA, all APPs showed significant and sustained elevation with A2M increasing the most.
- Haptoglobin and A2M assays now formatted for Advia 1650

Renal Biomarkers: Traditional clinical pathology parameters of renal injury

- Biomarkers of renal function
  - Primarily plasma markers of GFR
    - GFR most important clinically but it is a late change
- Biomarkers of renal injury
  - Urinary biomarkers
    - Routine urinalysis (including urine chemistry)
    - Novel rat urinary biomarkers (Lots of recent applications!)
      - Not discussed today

Biomarkers of renal injury (urinary enzymes, & proteins)
ILSI/HESI, PSTC, IMI, Academia, assay vendors….



Proximal Tubule
- GST-α
- β2-Microglobulin
- NAG
- Kim-1
- Cyr-61
- Lipocalin-2 (NGAL)
- Timp-1
- Clusterin
- EGF
- Osteopontin

Distal Tubule
- GST-μ
- Osteopontin
- Calbindin D28
- Timp-1
- Clusterin
- EGF

Collecting Duct
- RPA-1 (rat)
- TTF3
- Calbindin D28

Loop of Henle
- RPA-2 (rat)
- Osteopontin

Glomerulus
- Podocin
- Nephrin
- Collagen IV
- β2-Microglobulin (indirect)
- Osteopontin
- Cyr-61

Unspecific
- VEGF
- Cystatin C

- ualbumin, protein, Creatinine

Utilization of novel renal biomarkers in translational pharmacology

Kidney injury biomarkers – safety and efficacy assessment

Disease models:

- MLR/lpr lupus mouse
- Ischemia/ Reperfusion (I/R) acute kidney injury (AKI)
- BTBR ob/ob mouse

Assays for specific and non-specific kidney markers:

- NGAL
- Kim-1
- Cystatin C
- MCP-1
- NAG

- ClinPath:
  - serum: creatinine, BUN
  - urine: creatinine, protein, albumin

- Histology/ pathology

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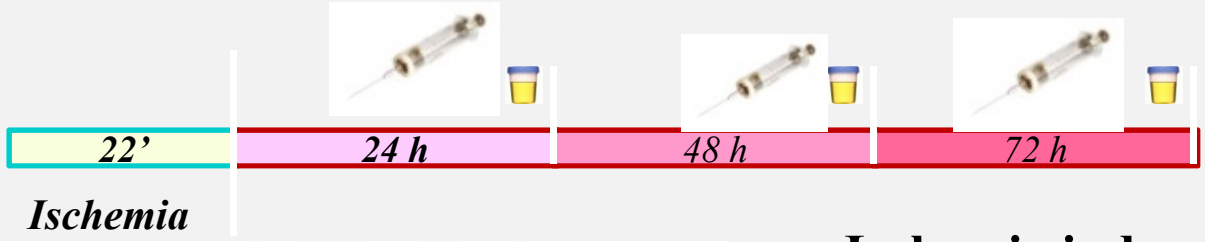
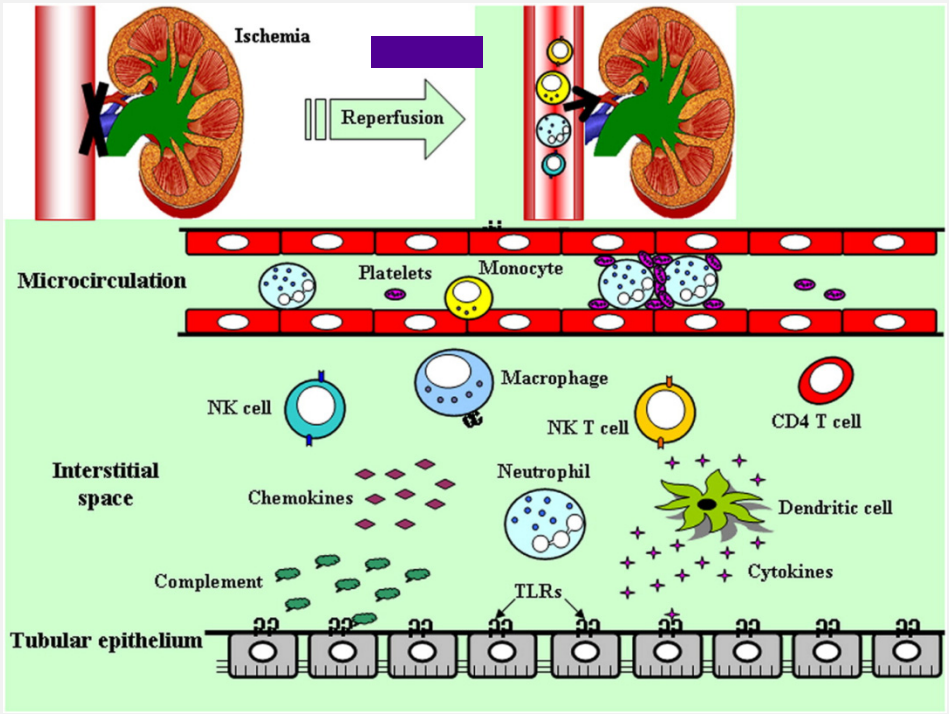
Acute Kidney Injury model



Ischemia



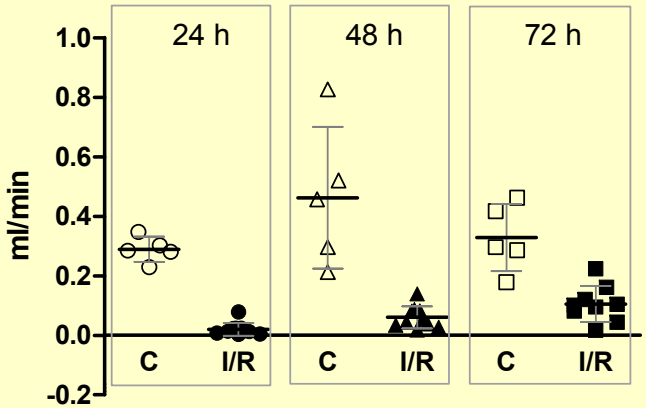
Ischemia induces vascular congestion, activation of resident dendritic cells, release of endogenous ligands and activation of TLRs

Restored blood flow during reperfusion period amplifies inflammatory process, oxidative stress and leads to further tissue damage

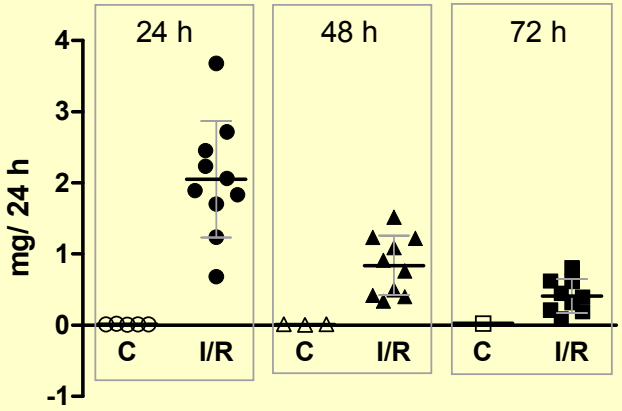
Modified from Clinical Immunology 2009; Vol.130; 41–50

Biomarkers of kidney function in I/R mouse

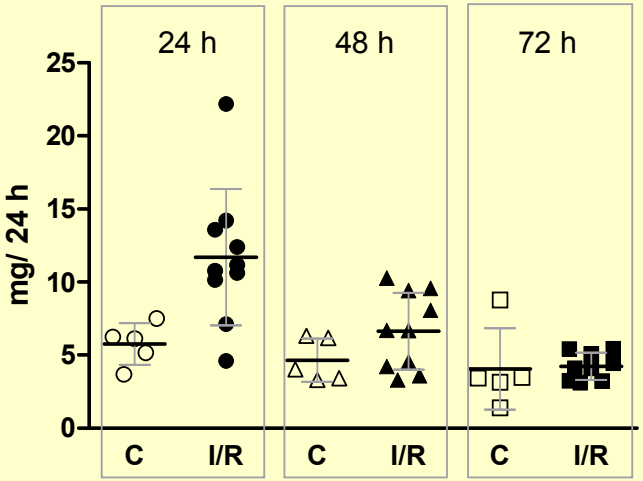
Creatinine clearance



Urine total albumin



Urine total protein



- ***Serum creatinine*** ↑
- ***Serum BUN*** ↑
- ***Urine NAG activity*** ↓

Creatinine Clearance:

$$\frac{\text{urine creatinine (mg/dL) x urine total volume (mL)}}{\text{serum creatinine (mg/dL) x collection time (minutes)}}$$

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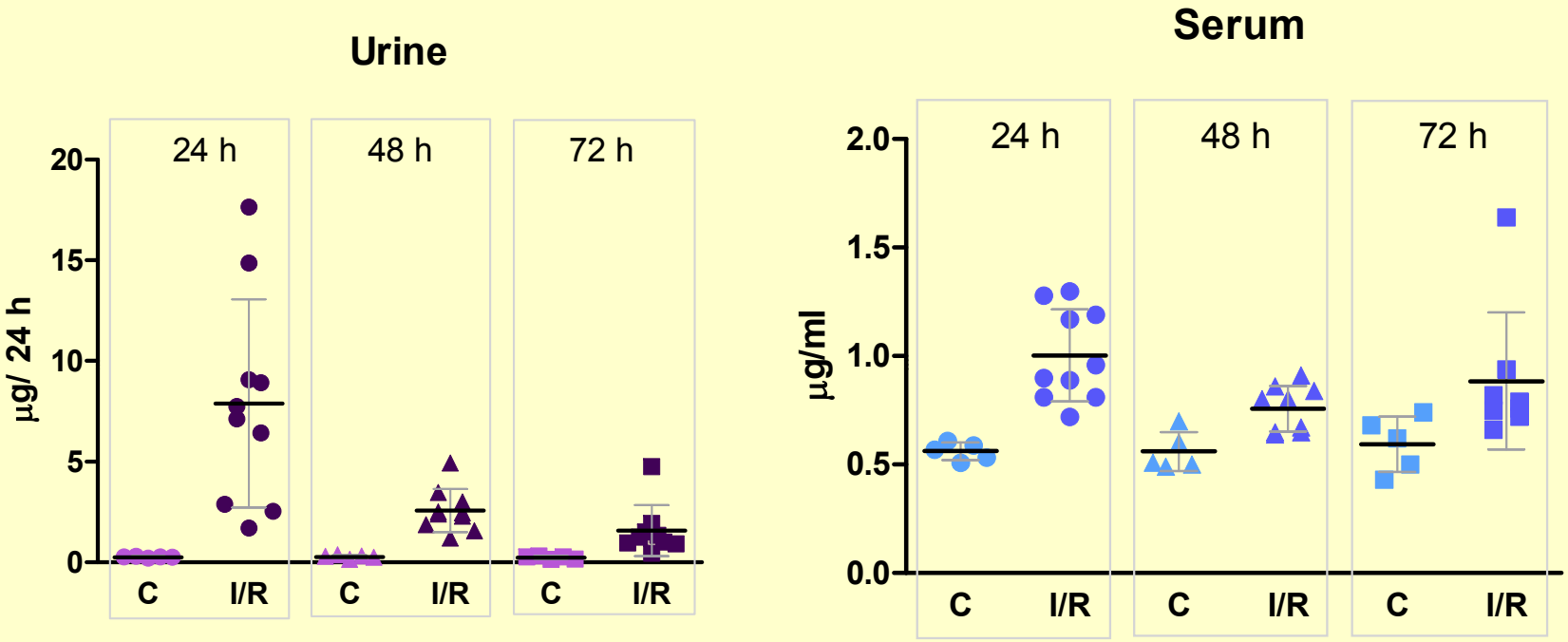
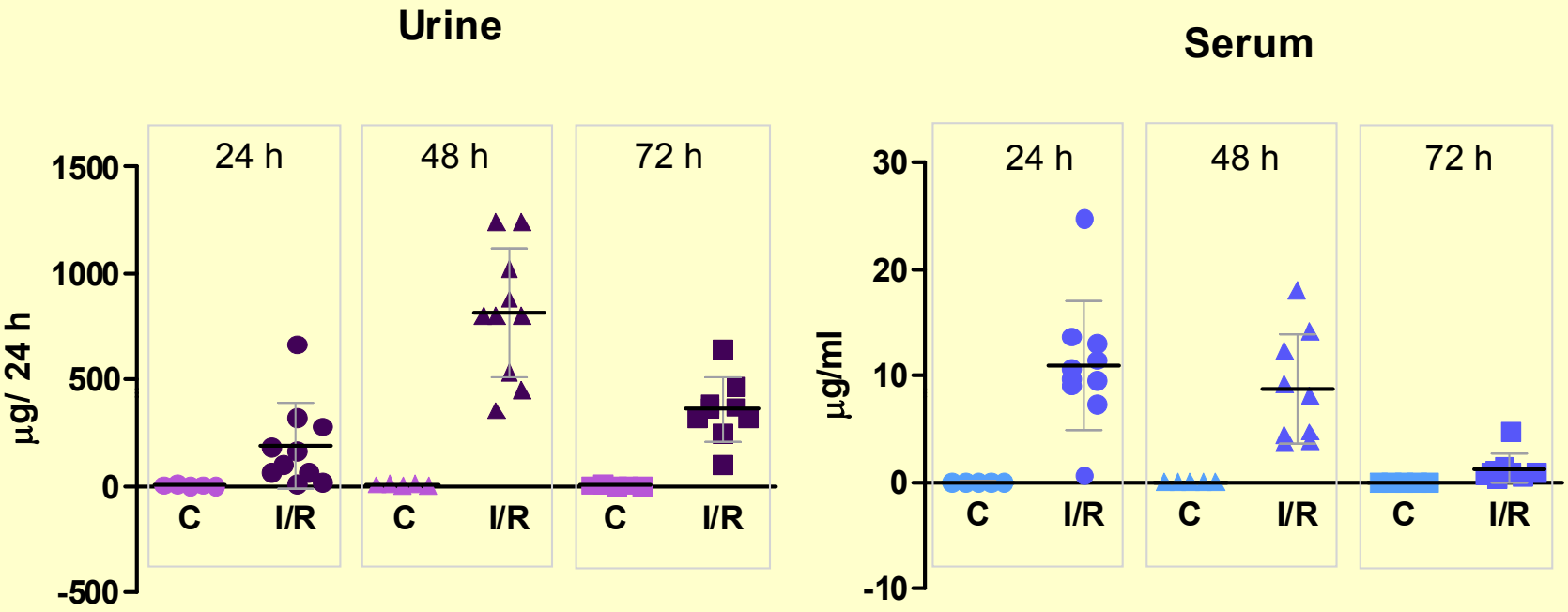
Urine Cystatin C – marker of tubular reabsorption



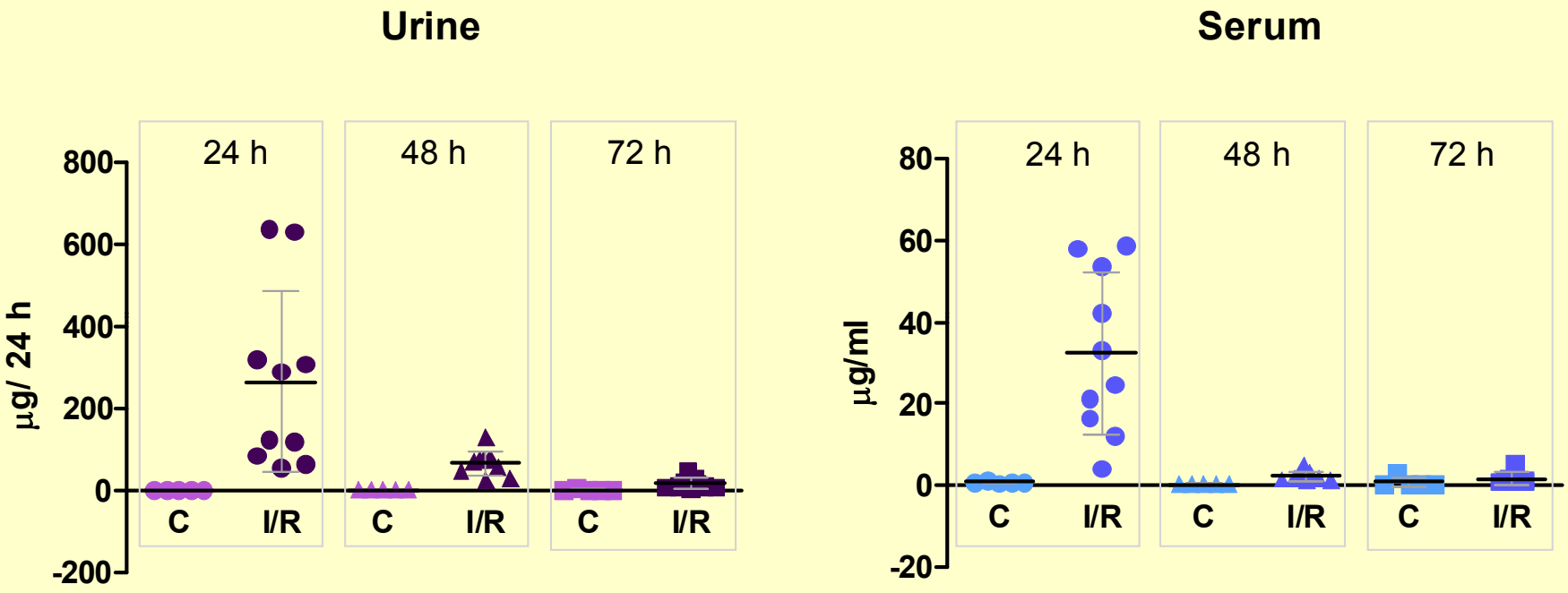
- ***Cystatin C:***
  - ✓ ***modest, ~2-fold increase in serum with greater induction in urine 24 h post injury***

Kim-1 – changes after ischemia/ reperfusion injury



- **KIM-1:**
  - ✓ **elevated in serum at 24 and 48 h post I/R injury**
  - ✓ **changes in urine levels follow changes in serum with the highest levels 48h post injury**
  - ✓ **greater magnitude of induction in serum than in urine in response to I/R**

NGAL – changes after ischemia/ reperfusion injury



•*NGAL:*

✓ *early marker of kidney injury, peaked within 24 h post I/R injury in serum and in urine*

✓ *recovery to normal level were faster in serum than in urine*

✓ *grater magnitude of induction in urine than in serum*

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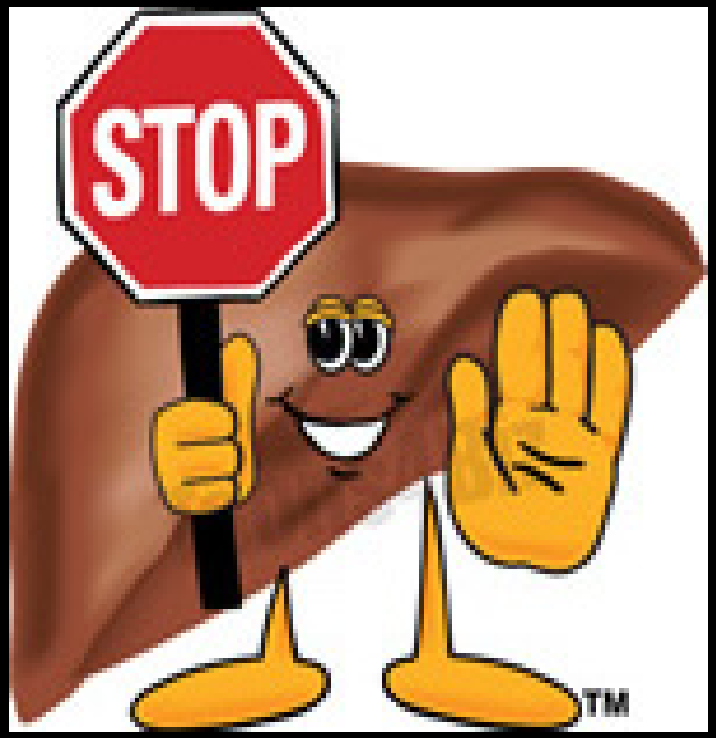
General Thoughts and Take Home Messages

1. Biomarker translation involves extensive planning
2. Clinical input provide needed perspective and attention
3. Emerging applications provide new options
4. Ask decision making questions
5. Link biomarkers to most applicable mechanisms and disease
6. Manage expectations
7. Knowing what to do with the information should be the first priority



Questions?